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Antigens

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The focus of this grant is to develop a vaccination strategy for patients with metastatic prostate cancer using DC-based vaccines loaded with normal prostate tissue-derived antigens. The key advantage of using normal prostate antigens, as opposed to using tumor-specific antigens, is that it will provide a universal and unlimited source of antigens for vaccination, and in combination with dendritic cells offers a powerful vaccination strategy that will be readily available to all patients with prostate cancer. Having established a TRAMP colony, protocols for measuring diseases progression in the TRAMP mice and protocols for measuring TRAMP C-1 tumor growth in mice we encountered two setbacks: 1) The TRAMP colony was severely decimated due to viral infection and 2) Generation of dendritic cells was severely compromised due to lack of access to a critical reagent. In response to these setbacks we have (1) regenerated the TRAMP colony-to be completed in March-April and (2) have developed a new, highly reliable method of generating high quality murine DC and demonstrated their functionality. We are currently testing the proposed vaccination protocols with normal prostate antigens in the TRAMP C-1 tumor model and will commence the parallel studies in the autochthonous TRAMP model when mice become available. Despite these setbacks, the experimental goals of the project will be met.

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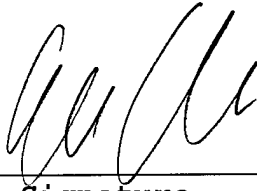
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## INTRODUCTION

The focus of this grant is to develop a vaccination strategy for patients with metastatic prostate cancer using DC-based vaccines loaded with normal prostate tissue-derived antigens. The key advantage of using normal prostate antigens, as opposed to using tumor-specific antigens, is that it will provide a universal and unlimited source of antigens for vaccination, and in combination with dendritic cells offers a powerful vaccination strategy that will be readily available to all patients with prostate cancer. The hypothesis underlying this, arguably unorthodox, proposal is that it will be possible to induce potent anti tumor immunity in the absence of clinical autoimmunity. While the hypothesis is supported by theoretical considerations and experimental evidence (discussed in the proposal) the primary objective of the studies proposed in this application is to test this hypothesis in a highly relevant animal model for metastatic prostate cancer. The studies described in the grant incorporate our extensive experience in DC vaccine development, including unique methods developed in our laboratory to augment the loading of DC with peptide antigens and the discovery that tumor RNA pulsed DC are highly effective stimulators of T cells and tumor immunity.

The specific goal of this project is to demonstrate in a murine model for prostate cancer that treatment of tumor bearing animals with syngeneic DC loaded with antigens isolated from prostatic tissue is capable of causing the regression or significant delay of metastatic spread in the treated animal without causing harmful autoimmune manifestations. If successful, these studies will set the stage for clinical trials using autologous DC pulsed with normal prostate antigens in patients with prostate cancer at high risk of relapse. The specific objectives of this proposal are:

- (1). To establish and calibrate the TRAMP murine model for prostate cancer.
- (2). To evaluate two promising vaccination strategies in the TRAMP model that were developed in our laboratory:
  - a). Using DC transfected with prostate tissue RNA
  - b). Using DC pulsed with peptide mixtures isolated from prostate tissue.

## BODY PROGRESS REPORT

### 1. Characterization of the TRAMP model.

The vaccination strategy described in this application will be evaluated in the murine TRAMP model (1). TRAMP is a transgenic line of C57BL/6 mice that develops histologic prostatic intraepithelial neoplasia by 18-22 weeks of age that progresses to adenocarcinoma with metastasis by 24-30 weeks of age. The TRAMP model is arguably the best murine model for prostate cancer as it closely resembles the human disease. The unique feature and special relevance of this animal model for our studies is that we will evaluate the impact of vaccination on autochthonous disease. We have established a colony of TRAMP mice from a breeding pair provided by Dr. Greenberg. We have previously determined the course of disease in our colony which progressed at a rate similar but not identical to what was described by Dr. Greenberg and his colleagues. We have also:

- I. In consultation with Dr. Greenberg and his colleagues we have developed the methodology for measuring and quantitating metastatic spread in the animal.

- II. Developed the methodology of surgically removing prostates and have collected >50 normal prostates for antigen preparation.

## 2. The TRAMP C-1 tumor models

We have calibrated the TRAMP C-1 (C-1) tumor model-determined a dose response curve in C57BL/6 mice, as a prerequisite for the immunotherapy studies. In our hands, C-1 tumors grew very slowly and somewhat erratically. We found that implantation in the ear and use of matrigel improves the efficiency of take, rate of tumor progression and constancy.

## 3. Setbacks

In the course of our studies during year #1 of funding we have encountered two serious setbacks,

a) The TRAMP colony was decimated due to an outbreak of viral infection in the animal facility and b) We encountered serious difficulties in generating murine dendritic cells due to lack of a crucial reagent.

a). *The TRAMP colony.* A breeding pair of TRAMP mice were obtained originally from Dr. Greenberg and a colony was established and evaluated as described above. Due to viral infection in our animal facility, which occurred 5 month ago, the TRAMP colony was decimated. The virus was not characterized and was manifested as loss of male fertility (Breeding pairs consist of a male TRAMP and wild type C57BL/6 females of which 25% of offspring are male TRAMP). Dr. Greenberg, the originator of the TRAMP mouse has experienced similar problems (personal communication). We were, however, able to rescue two fertile males and establish breeding colonies. We are now half way through rebuilding the colony to its original size which should be completed in March-April.

b). *Murine dendritic cell (DC) generation.* DC are a centerpiece of the proposed studies as they constitute the vehicle used to vaccinate the animal with the prostate antigens. Dendritic cells are generated from bone marrow of mice in the presence of murine GM-CSF (2). Murine GM-CSF is commercially available but is prohibitively expensive. We have originally obtained GM-CSF for DC preparation as a gift from Amgen Corp. but subsequently have switched (by necessity) to Immunex as a source of GM-CSF. Unfortunately, the recent lots of Immunex GM-CSF did not support effective DC generation (see below). This was most unfortunate since DC biology and DC-based vaccination is a centerpiece of our research program and hence the inability of generating DC has impacted a significant portion of our research activities, in addition to this project.

Since three consecutive lots of Immunex GM-CSF were not active, it became imperative to redirect our efforts in developing an alternative strategy for generating murine DC, for this and other studies ongoing in our laboratory. We are relieved to report that our efforts were successful and the program is therefore back on track. Here is briefly a summary of what we have done.

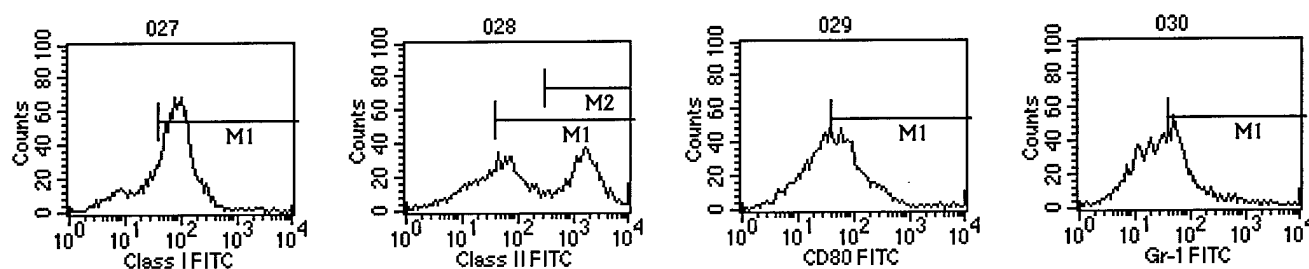
I). Having exhausted non commercial sources of GM-CSF, we established a cell line secreting GM-CSF and used the conditioned media as a source of GM-CSF to generate DC. The GM-CSF cell line was established by first constructing a GM-CSF expression plasmid which was introduced into F10.9 cells, a fast growing tissue culture adapted melanoma cell line. Figure 1, upper panel shows that using F10.9 derived GM-CSF we were able to generate DC albeit their phenotypic appearance was compromised as indicated by the moderate to high expression of granulocyte markers (Gr-1) and both low and high MHC class II expressing cells.

II). We therefore embarked on a series of attempts to modify the original DC generation protocol (2) to make it compatible with the new source of GM-CSF. The issue being that the Amgen and Immunex

GM-CSF were recombinant product and the new source of GM-CSF is provided as conditioned media which is likely to contain other, growth or inhibitory, factors. In the original protocol,

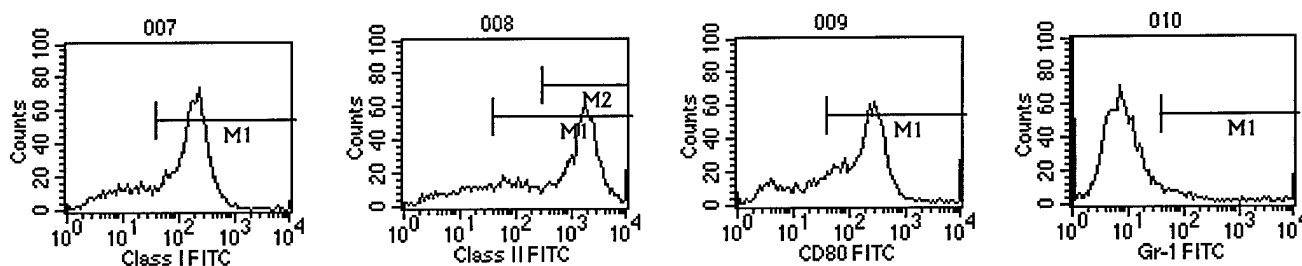
the bone marrow progenitors are cultured in bovine serum containing medium + GM-CSF. With this in mind, we explored methods which dispense with the use of foreign serum components.

#### Day 9 BMDCs generated in medium containing FBS and F10.9-derived GM-CSF



Day 9 yield: 8.9% of plated marrow progenitors  
Day 9 viability by trypan blue: 98.4%

#### Day 9 BMDCs generated in serum-free medium containing F10.9-derived GM-CSF



Day 9 yield: 10.3% of plated marrow progenitors  
Day 9 viability by trypan blue: 91.0%

**Figure 1. Flow cytometry of bone marrow DC.** Murine DC were generated from the bone marrow by established procedures (2) with F10.9 derived GM-CSF and analyzed by flow cytometry for the expression of class I, class II, B7-1 (CD80) and Gr-1, a granulocyte-specific marker. Upper panels: DC were generated in RPMI-1640 medium with fetal bovine serum (FBS). Lower panels: DC were generated in Sigma serum-free medium.

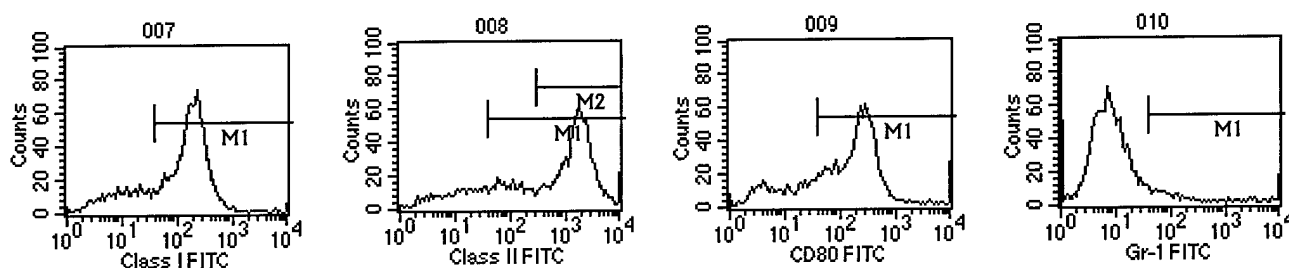
We have identified a medium composition from Sigma which permitted the generation of DC in the absence of medium. Figure 1, bottom panels, show the phenotype of the serum-free DC derived in the

presence of GM-CSF conditioned media. Note by comparison the high yield of class II<sup>high</sup> cells and the absence of granulocytes.

However, whereas the phenotype of the DC was excellent, their viability was limited. We therefore tested and found that addition of a small amount of syngeneic murine serum (0.3%) was sufficient to improve viability to previous levels.

We then compared the F10.9 derived GM-CSF to Immunex GM-CSF in generating DC by the new protocol and as shown in Figure 2, the Immunex GM-CSF was still significantly suboptimal.

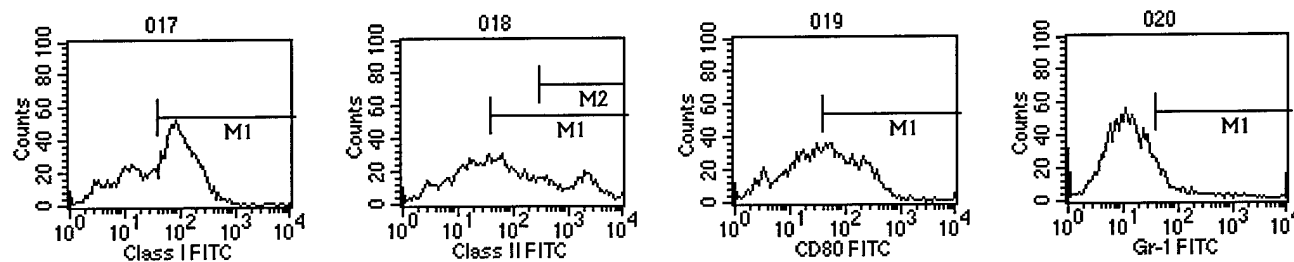
#### Day 9 BMDCs generated in serum-free medium containing F10.9-derived GM-CSF



Day 9 yield: 10.3% of plated marrow progenitors

Day 9 viability by trypan blue: 91.0%

#### Day 9 BMDCs generated in serum-free medium containing Immunex GM-CSF



Day 9 yield: 6.3% of plated marrow progenitors

Day 9 viability by trypan blue: 70.3%

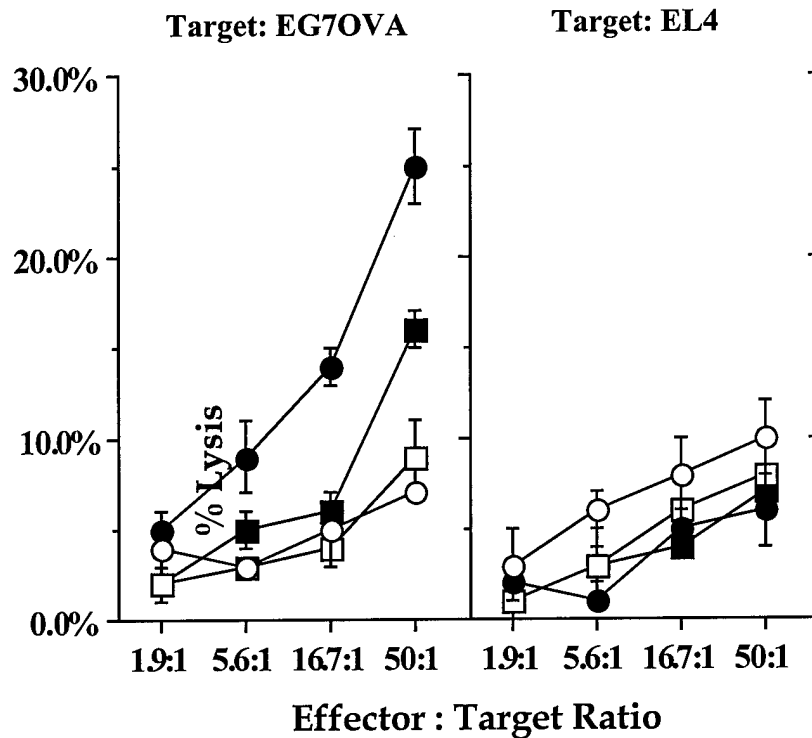
**Figure 2. Flow cytometry analysis of murine DC-Comparison of F10.9 derived GM-CSF and Immunex GM-CSF.** (See Figure 1 legends for details)

To confirm the functionality of the DC we performed a standard CTL experiment-measuring the ability of OVA peptide pulsed DC to stimulate an OVA CTL in mice. As shown in Figure 3, DC generated in the presence of F10.9 derived GM-CSF were superior to DC generated in the presence of Immunex GM-CSF in priming a CTL response.



### Immunizations

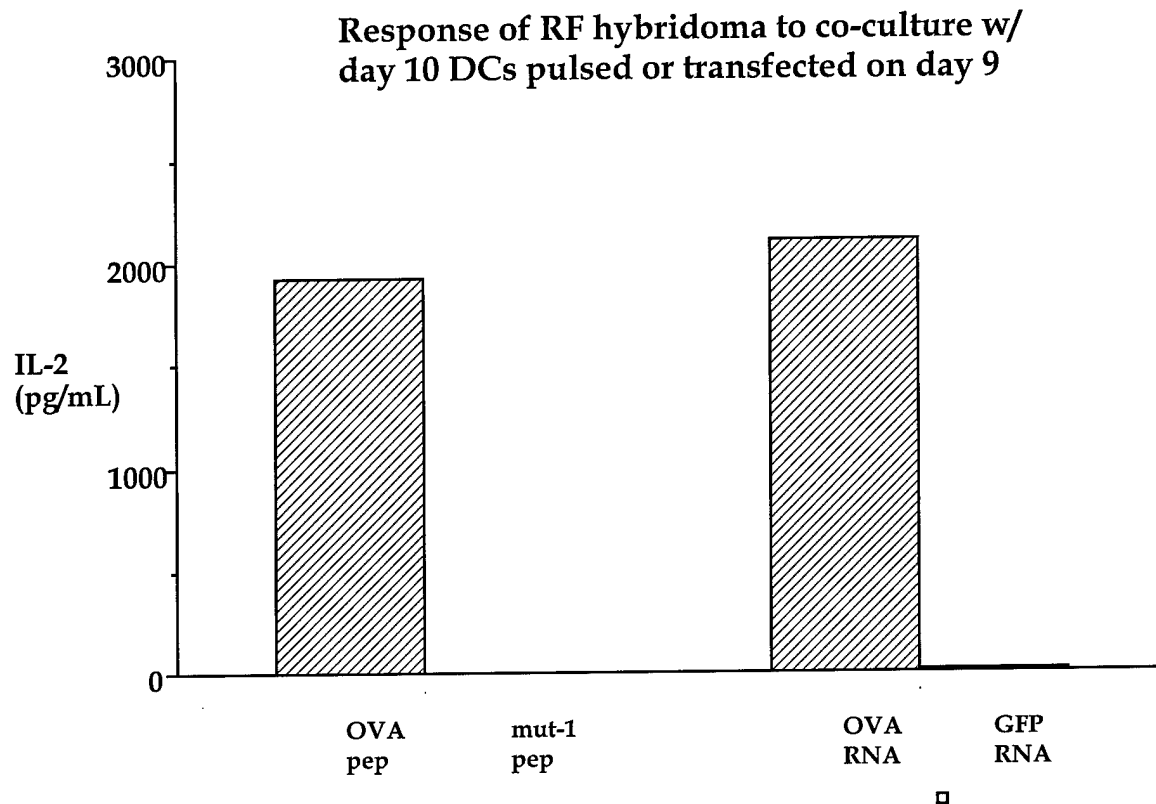
- DC-OVA peptide (F10.9 GM-CSF)
- DC-mut-1 peptide (F10.9 GM-CSF)
- DC-OVA peptide (Immunex GM-CSF)
- DC-mut-1 peptide (Immunex GM-CSF)



**Figure 3: Priming of a CTL response in mice using DC generated with F10.9 derived GM-CSF and Immunex GM-CSF.** DC prepared in mouse serum and either F10.9 derived GM-CSF or Immunex GM-CSF were pulsed with OVA peptide and injected into C57BL/6 mice. Splenocytes were removed 5 days later, stimulated in vitro with OVA-expressing E.G7-OVA cells and assayed for OVA-specific CTL using E.G7-OVA or EL4 targets (3, 4).

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To show that the DC generated in F10.9 derived GM-CSF can be sensitized with peptide or RNA-the form of antigens that will be used in this project-we have shown that OVA peptide pulsed or OVA RNA transfected DC were comparatively effective at stimulating an OVA-specific hybridoma to secrete IL-2. (Figure 4).



**Figure 4: Stimulation of an OVA-specific hybridoma using DC pulsed with OVA peptide or transfected with OVA RNA.** DC were generated in murine serum and with F10.9 derived GM-CSF. DC were incubated with a hybridoma specific to a class I restricted OVA peptide for 24h and supernatant was assayed for IL-2 secretion (5).

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In summary, we have successfully resolved a serious technical problem and have developed an alternative, inexpensive, and perhaps superior, method of generating murine DC. This work is now summarized for publication.

#### 4. Ongoing studies and plans for next year

While we have clearly lost precious time, we hopefully came out stronger in having developed a more potent dendritic cell. In order to expedite the accomplishment of the stated goals of the project as delineated in the proposal and the Statement of Work, we are establishing a parallel track of examining the antimetastatic impact of vaccination in the TRAMP C-1 tumor model and the TRAMP mice.

Having established the growth pattern of TRAMP C-1 tumor cells in mice we are gearing up to vaccinate mice with TRAMP C-1 derived peptide and RNA transfected DC and challenge with tumor cells. Following this experiment, we will use peptide or RNA isolated from the normal prostates (Year 1:Task 4 and 5)

With the completion of rebuilding the TRAMP colony-should be completed in March-April-we will begin the vaccination protocols in the TRAMP male mice as described in the Statement of Work, Year 2:Task 6-8.

I am confident that we will be able to accomplish the main goals of the project within the specified period

## **KEY RESEARCH ACCOMPLISHMENTS**

1. Characterized the disease pattern of TRAMP mice and established the know-how of evaluated disease progression.
2. Established a dose response curve of TRAMP C-1 tumor cells.
3. Have developed an alternative and reliable method of generating dendritic cells for vaccination.

## **REPORTABLE OUTCOMES-N/A**

## **CONCLUSIONS-N/A**

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## **APPENDICES-NON**